EXHIBIT 11

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Clinical Protocol

Immunotherapy of Malignancy by *In Vivo* Gene Transfer into Tumors

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1.0 Introduction

1.1 Description of Revisions

This proposal was approved by the Human Gene Therapy Subcommittee of the RAC on November 21, 1991. Several suggestions were made during the review for modification of the protocol. A summary of these modifications are indicated in bold on the original application and summarized below:

1. Clarification regarding the timing of tumor biopsy was suggested, including the time of biopsy after gene transfer and additional biopsies depending on the response of the injected tumor nodule. This information has been described in the revised protocol (Sections 6.1, 6.2, and 9.0). Briefly, tumor nodules will be biopsied 1 week after initial gene transfer, and, subsequently, at the time of repeat injection. Should the cutaneous nodule disappear after initial treatment, an additional biopsy will be made at the site of injection 2 weeks after introduction of the recombinant gene. If the nodule is larger in size, an additional biopsy will be performed. In each case, the status of HLA-B7 gene expression will be analyzed. The presence of nucleic acid will be confirmed by PCR, and the production of recombinant HLA-B7 protein will be confirmed by immunostaining. The induction of specific immune response against HLA-B7 or against the tumor will be assessed with the cytolytic T cell assay. The criteria for successful response to treatment are also further elaborated (Section 11.1).

2. It was suggested that a postmortem examination be described in the protocol, with emphasis on pathologic analysis of tissues and PCR analysis of DNA from testes or ovaries. Although an autopsy cannot be required as a precondition for entry into this study, this issue will be discussed with each patient. If possible, such analyses will be performed on all patients. This has been described in the revised protocol (Section 6.5).

3. Patients will be sensitized to the HLA-B7 antigen likely making them ineligible for transplantation with organs from donors with this antigen (~15% of donors). This information has been included in the revised "Informed Consent" (Section 15).

4. The vector sequence, together with all potential open reading frames, have been provided on computer disk. No open reading frames encoding oncogenes have been detected, and these open ready frames are shown (Appendix V). In addition, this vector has been used in 3T3 transformation assays, and does not cause cell transformation. Although three vectors have been prepared and characterized, vector II is expressed best both in human melanomas and other cells and is proposed for use in this study.

5. Clarification of the integration status of the plasmid DNA was requested. In general, we find that plasmids which are not linearized prior to lipofection are expressed transiently, in contrast to linearized plasmids which stably integrate into host cells with higher frequency. We are currently performing Southern blot analysis of episomal or chromosomal DNA to determine the percentage of plasmids which remain episomal or integrate after lipofection.

6. Confirmation was requested that melanoma, and not adenocarcinoma, will be treated. Although it is a potential target of future studies, adenocarcinoma will not be included for the initial phase I studies. A confirmatory letter has been sent to our local IRB.

7. Additional studies have been taken to document the mechanism of tumor protection in this model system. Recent studies have been performed using direct gene transfer in nude mice with the MCA 106 fibrosarcoma. The introduction of H-2K^{S} into tumors within these animals does not result in any tumor protection, further substantiating the T cell dependency of this response. Finally, an HLA-B7 expression vector containing a deletion in the $\alpha 1$ and $\alpha 2$ domains of the class I MHC genes does not confer an anti-tumor response. These results are described in the revised "Preliminary Data" section (Section 13), as are additional data regarding expression in human melanoma cells and systemic tumor immunity presented during the November 21 meeting.

This proposal is now presented in modified form to address the concerns of the Human Gene Therapy Subcommittee. The most recent modifications are indicated in bold for the convenience of the reviewers. Previous modifications are summarized in Section 24, Appendix VI.

1.2 Background

Immunotherapy has shown promise as an adjuvant approach to the treatment of malignancy. Both cytolytic T cells and lymphokines can facilitate tumor cell destruction, and strategies to enhance tumor regression by administration of cytokines or tumor infiltrating lymphocytes have shown efficacy in animal models and human trials. We have developed a model for the immunotherapy of malignancy, based on a novel method to stimulate an immune response against tumor cells by genetic modification *in vivo*. This approach differs from previous methods in which tumor cells are propagated, modified, and selected *in vitro*. We have recently shown that recombinant genes can be introduced and expressed at specific anatomic sites *in vivo* by direct gene transfer into vessels. This method has been adapted to deliver recombinant genes to tumor cells *in vivo*. We have introduced genes encoding a highly immunogenic molecule, an allogeneic class I major histocompatibility complex (MHC) glycoprotein, into transplantable mouse tumors. When the foreign MHC gene has been delivered into tumors *in vivo*, it induces cytolytic T cells against this antigen and has produced partial tumor regressions. More importantly, this treatment elicits a cytolytic T cell response against parental unmodified tumor cells. This approach therefore provides an alternative approach to the immunotherapy of malignancy. It can be used alone or in combination with other genes, including cytokines, to cause tumor regression.

In this study, a phase I clinical trial is proposed. The safety of this method and appropriate dosage in humans will be tested. Recombinant gene expression *in vivo* will be confirmed, and the specificity and mechanism of immune rejection will be defined. In subsequent phases, this response will be augmented by preimmunization and administration of cytokines, including tumor necrosis factor-α, interferon-γ, or interleukin-2, or used in combination with adoptive transfer or TIL therapy. These studies will provide an alternative strategy for the immunotherapy of malignancy and allow definition of the mechanism of immune rejection of tumor cells. Adaptations of this method may also eventually be applied to the treatment of other human diseases.

1.3 Direct Gene Transfer In Vivo

The conventional approach to immunotherapy is based on the modification of T lymphocytes or tumor cells *in vitro*, followed by reintroduction *in vivo*. Limitations of this approach include 1) the need to establish a cell line from each experimental subject and to avoid allogeneic tissue rejection, 2) concerns about alteration of the phenotype of cells propagated in tissue culture, 3) the outgrowth of aberrant transformed cells, and 4) the time and effort required to establish cell lines, introduce genes, select for a relevant phenotype and characterize the cells prior to reintroduction into animals.

We have therefore developed an alternative approach in which recombinant genes are introduced directly into cells *in vivo*. Introduction of recombinant genes into cells *in vivo* provides a powerful method to study their effects in a relevant setting in humans. This approach has been utilized by our laboratory to study the effect of recombinant gene expression within transduced vascular cells in a localized arterial segment in several animal models. We have used two delivery vehicles, retroviral vectors and DNA/liposome-mediated transfection. Of these two approaches, the DNA/liposome method can be more readily adapted to the treatment of human disease. Initial studies focused on the use of retroviral vector delivery. An amphotropic retroviral vector was used to infect porcine vascular cells *in vivo* (1). Supernatant from a β-galactosidase transducing retroviral producer cell line (2), proven to be free of replication competent virus (10^5–10^6 virus particles/ml), was introduced into a localized segment of the illofemoral artery in pigs using a double balloon catheter, and expression of the marker gene was confirmed up to five months following transduction. Immunohistochemical staining demonstrated that β-galactosidase was expressed within endothelial cells and vascular smooth muscle cells. No evidence of replication-competent virus was detected, and gene expression was localized to the target site (1).

DNA/liposome complexes have provided an alternative method for direct gene transfer into tissues *in vivo* (3,4). One advantage of this method is that any plasmid containing a gene in a eukaryotic expression vector with appropriate regulatory sequences can be easily utilized. Liposomes can obviate the need to synthesize a retroviral vector plasmid, establish subclones of retroviral producer cell lines, test them for viral titer, and assess the presence of replication-competent helper virus. We have employed liposomes complexed to plasmid DNA, containing β-galactosidase or other genes, to transduce localized arterial segments. This method of delivery also provides high levels of recombinant gene expression localized to specific sites *in vivo*. These experiments employing direct gene transfer *in vivo* are described (1).

We have also utilized cell-mediated gene transfer to deliver genes to specific sites *in vivo*. Vascular smooth muscle cells or endothelial cells, transduced *in vitro* with E. coli β-galactosidase, have provided a vehicle to deliver this and other recombinant genes into localized arterial segments (5,6). We infected either endothelial or vascular smooth muscle cell lines, established from Yucatan minipigs, with the BAG retroviral vector (7) and selected populations of transduced cells which have been introduced *in vivo*.

These studies demonstrate that recombinant genes can be expressed *in vivo* by several strategies. Cell-mediated gene transfer can be achieved using vascular cells (5,6). This method is now being used to study the effects of expressing recombinant genes for cytoplasmic, membrane bound, or secreted protein products within living animals. Second, direct introduction of recombinant genes *in vivo* has been achieved (1) and provides a way to modify cells at specific sites *in vivo*.

1.4 Immunotherapy of Malignancy

The immune system not only provides protection against a variety of pathogens, but also appears to contribute to the surveillance and destruction of neoplastic cells. Several cellular and humoral immune effectors inhibit tumor cell growth. Cellular mediators with anti-tumor activity include MHC-restricted cytotoxic T cells, reviewed in (8), natural killer (NK) cells (9,10) and lymphokine-activated killer (LAK) cells (11). Cytolytic T cells which infiltrate tumors have been isolated and characterized (12). These tumor infiltrating lymphocytes (TIL) selectively lyse cells of the tumor from which they were derived (13,14). Macrophages can also kill neoplastic cells through antibody-dependent mechanisms (15,16), or by activation induced by substances such as BCG (17).

Cytokines are important in the anti-tumor response through direct action on tumor cells and by activating cellular components of the immune system. Tumor necrosis factor-α (TNF-α) (18) and lymphotoxin (19) have direct effects on neoplastic cells resulting in cell death. Interferon-γ (IFN-γ) induces a marked increase in surface expression of class I MHC molecules (20,21) and synergizes with TNF-α in producing this effect (22). Colony stimulating factors such as G-CSF and GM-CSF activate neutrophils and macrophages to lyse tumor cells directly (23). Interleukin-2 (IL-2) activates Leu-19$^+$ NK cells to generate lymphokine activated killer cells (LAK) capable of lysing autologous, syngeneic or allogeneic tumor cells but not normal cells (11,24,25). The LAK cells lyse tumor cells without preimmunization or MHC restriction (26). Interleukin-4 (IL-4) also generates LAK cells and acts synergistically with IL-2 in the generation of tumor specific killer cells (27).

Although the immune system can be stimulated to mediate tumor cell destruction, the vast majority of malignancies arise spontaneously in immunocompetent hosts. This observation suggests that tumor cells escape these host defenses by mechanisms which are incompletely defined. It has been suggested that evolution of tumor cells provides a proliferative advantage to successively less immunogenic clones (28). Deficient expression of class I MHC molecules limits the ability of tumor cells to present antigens to cytotoxic T cells. Freshly isolated cells from naturally occurring tumors frequently lack class I MHC antigen completely or show decreased expression (29–33). Reduced class I MHC expression is thought to facilitate growth of these tumors when transplanted into syngeneic recipients. Several tumor cell lines which exhibit low levels of class I MHC proteins become less oncogenic when expression vectors encoding the relevant class I MHC antigen are introduced into them (34–38). In some experiments, tumor cells which express a class I MHC gene confer immunity in naive recipients against the parental tumor (35,36). The absolute level of class I MHC expression, however, is not the only factor which influences the tumorigenicity or immunogenicity of tumor cells. In one study,

mouse mammary adenocarcinoma cells, treated with 5-azacytidine and selected for elevated levels of class I MHC expression did not display altered tumorigenicity compared to the parent line (39).

A variety of methods can be used to augment the immune response against tumor cells. These include systemic administration of IL-2 (40), or IL-2 with LAK cells (41,42). Clinical trials using tumor infiltrating lymphocytes are currently in progress (43). Recently, several studies have examined the tumor suppressive effect of lymphokine production by genetically altered tumor cells. The introduction of tumor cells transfected with an IL-2 expression vector into syngeneic mice stimulated an MHC class I restricted cytolytic T lymphocyte response which protected against subsequent rechallenge with the parental tumor cell line (44). Expression of IL-4 by plasmacytoma or mammary adenocarcinoma cells induced a potent anti-tumor effect mediated by infiltration of eosinophils and macrophages (45). These studies demonstrate that cytokines, expressed at high local concentrations, are effective anti-tumor agents.

We propose an alternative approach to stimulate an anti-tumor response, through the introduction of an allogeneic class I MHC gene into human tumor cells. The antigenicity of tumor cells has been altered previously by the expression of viral antigens through infection of tumor cells (46–50), or expression of allogeneic antigens introduced by somatic cell hybridization (51,52). Allogeneic class I MHC genes have been introduced into tumor cells by transfection and subsequent selection *in vitro*. These experiments have produced some conflicting results. In one case, transfection of an allogeneic class I MHC gene (H-2L^d) into an H-2^b tumor resulted in immunologic rejection of the transduced cells and also produced transplantation resistance against the parent tumor cells (53). In another instance, transfection of H-2^b melanoma cells with the H-$2D^d$ gene did not lead to rejection (54), however increased differential expression of H-2D products relative to H-2K may have affected the metastatic potential and immunogenicity of tumor cells (55). The effects of allogeneic H-2K gene expression in tumor cells was examined in another study (56). Several subclones which were selected *in vitro* and expressed an allogeneic gene were rejected in mice syngeneic for the parental tumor line, however, other subclones did not differ from the parental, untransduced line in generating tumors. This study suggests that conflicting results may be obtained based on clone-to-clone variation in *in vivo* growth and tumorigenic capacity, suggesting that other modifications of these cells, caused by transfection or the subcloning procedure, affected their tumorgenicity. These types of clonal differences would likely be minimized by transducing a population of cells directly *in vivo*.

Because the H-2K class I MHC antigen is strongly expressed on most tissues and can mediate an allogeneic rejection response, we chose it in our animal model studies designed to enhance the immunogenicity of tumors *in vivo*. These studies extend previous efforts to modify tumor cells by developing a system for the direct introduction of genes into tumors by *in vivo* infection using retroviral vectors or by DNA/liposome mediated transfection. This technology can also be used to deliver specific recombinant cytokines into the tumor microcirculation and to understand the immunologic basis for tumor rejection *in vivo*.

2.0 Objectives

The immune system can provide protection against cancer and may play an important role as an adjuvant treatment for malignancy. Lymphokine activated killer cells (LAK) and tumor infiltrating lymphocytes (TIL) can lyse neoplastic cells and produce partial or complete tumor rejection. Expression of cytokine genes in malignant cells has also enhanced tumor regression. Because current strategies to stimulate an immune response against tumor cells often fail to eradicate tumors, an important goal of immunotherapy is to improve upon current techniques and understand the mechanisms of immune recognition.

In this study, a novel approach to enhance the immune response against tumors will be explored by the introduction of recombinant genes directly into tumor cells *in vivo*. Traditionally, gene transfer techniques have focused on modification of tumor cells *in vitro*, followed by transfer of modified cells. Such approaches subject these cells to selection and different growth conditions from those which act *in vivo*. Because they also require that cell lines be established for each malignancy, adaptability to human disease is more difficult.

We have developed a model for the immunotherapy of malignancy using a gene encoding a transplantation antigen, an allogeneic class I major histocompatibility complex (MHC) antigen, which will be introduced into human tumors *in vivo* by DNA/liposome transfection. In animal studies, we find that the expression of allogeneic MHC antigens on tumor cells stimulates immunity against both the allogeneic MHC gene on transduced cells as well as previously unrecognized antigens in unmodified tumor cells. The introduction of an allogeneic MHC gene directly into tumors *in vivo* has induced partial tumor regressions, as well as the specific cytotoxic T cell response to other antigens described above. In murine systems, we continue to optimize this gene delivery approach and characterize the cellular basis of this protective response, relevant cytokines which mediate this effect, and the nature of tumor specific antigens recognized by activated cytolytic T cells. This approach is also being used to introduce other recombinant genes with anti-tumor effects, such as tumor necrosis factor-α, into tumors.

Because this approach employs direct gene transfer *in vivo*, it can be applied easily in a clinical setting to spontaneously arising tumors, alone or in combination with cytokines or other adjuvant treatments, including TIL cells, to augment tumor immunity. In this study, we propose a phase I clinical trial to evaluate the safety and appropriate dosage to introduce a recombinant HLA-B7 antigen into human tumors *in vivo*. Recombinant gene expression *in vivo* will be documented, and the specificity and mechanism of the immune response will be characterized. Escalating treatment regimens will be used and tumor growth evaluated. These studies will define the safety of this approach to the immunotherapy of malignancy and may provide therapeutic effects for patients. Adaptations of this approach might also prove useful in the treatment of other human diseases. Specifically, we plan:

1) To establish a safe and effective dose to introduce a recombinant HLA-B7 gene into tumors by DNA/liposome-mediated transfection in living human subjects.

2) To confirm expression of this class I MHC gene introduced directly into tumor cells *in vivo*.
3) To analyze the specificity of the immune response against this antigen *in vivo* by analyzing cellular and humoral immunity.

3.0 Selection of Patients

Patients will be carefully selected based on consideration of their past medical history and present status. The referring physician, the attending physician, and surgical oncology or the relevant clinical department, the patient, and family members will make a joint decision regarding the appropriate treatment with conventional therapy. If surgery, chemotherapy, or radiation are deemed unlikely to provide further benefit to the patient, the opportunity to pursue this experimental protocol will be offered to the patient. The following criteria will be used to select appropriate patients for study:

3.1 Criteria for Patient Eligibility

3.1.1 Patients who are HLA-B7 positive will not be studied.
3.1.2 Patients with cutaneous tumor nodules will be studied. These will most likely be limited to patients with malignant melanoma with cutaneous lesions, as well as distant metastases.
3.1.3 The patient's disease will be unresponsive to standard modes of treatment.
3.1.4 Patient must be greater than 18 years of age.
3.1.5 The patient must not have antibodies to the Human Immunodeficiency Virus.
3.1.6 Patient must either be postmenopausal or have undergone tubal ligation, vasectomy, or orchiectomy.
3.1.7 The patient must be able to provide informed consent.

3.2 Criteria for Patient Ineligibility

3.2.1 Patients with active autoimmune disease.
3.2.2 Patients who have a positive antibody to HIV.
3.2.3 Patients with active hepatitis, chronic or acute.
3.2.4 Patients with diabetes mellitis who are not controlled by medical treatment.
3.2.5 Psychiatric illness which makes compliance to the clinical protocol unmanageable.
3.2.6 Patients on high dose glucocorticosteroids.
3.2.7 Patients who undergo an alternative mode of anticancer treatment will not be accepted within 4 weeks of the last treatment.

4.0 Clinical Evaluation Prior to Treatment

As described above, patients will be selected based on their past medical history and clinical course. Patients must have histologically confirmed metastic melanoma which is unresponsive to standard curative or palliative measures. Patients with expected survival times of 1 year or less will be chosen. The patients must meet the standard inclusion and exclusion criteria described above. In addition, the chosen patients must show no signs of active systemic infection or major cardiovascular or respiratory disease. The following laboratory values will be used as guidelines as enrollment:

1. The bilirubin should be less than 2 mg/dl. The platelet count should be greater than 100,000/mm^3. The white blood cell count should exceed 3,000/mm^3. The creatinine should be less than 2.0 mg/dl.
2. HLA typing must indicate that the patient is HLA-B7 negative.
3. HIV antibody titers will be measured.

5.0 Stratification and Randomization

Not relevant.

6.0 Nature of Procedures or Therapeutic Agents

6.1 Study Design

Patients will be admitted to the Clinical Research Center at The University of Michigan Medical Center after the relevant eligibility criteria have been met. The pre-treatment evaluation will be performed as described in Section 9. Patients undergoing the direct gene transfer protocol will have serum samples removed for analysis as described in Section 9. The tumor nodule to be injected will be identified and its borders measured prior to injection. In addition, this nodule and other control (untreated) nodules will be imaged by CT immediately prior to the procedure, and its size quantitated. The skin overlying the tumor nodule will be sterilized and anesthetized using 1% lidocaine. Immediately prior to injection with the DNA liposome complex, a needle biopsy of the nodule will be performed if needed. Tissue will be stored as frozen sections for further immunohistochemical analysis and PCR. For gene transfer, a 22–26 gauge needle will be used to inject the DNA/liposome complex which will be prepared as follows: 15 minutes prior to delivery, 1–10 μg of plasmid DNA (20 μg/ml) is diluted to 200 μl of lactated-Ringer's solution at room temperature and added to 5–10 μl (10–20 nM) of DC-Chol liposome solution (2 mM). Each component will be stored separately in sterile vials and certified as acceptable by the FDA. The solution is left at room temperature for 5–15 minutes and 0.5 of sterile lactated-Ringer's is added to the liposome DNA solution. The optimal composition of the DNA/liposome complex will be established for each batch by titration of DNA concentration and liposome concentration independently on murine melanoma and Hela cells *in vitro*, and confirmed by direct injection into murine melanoma or other tumors *in vivo* prior to use. Each component, the liposome preparation, and the DNA, will be tested for contaminants and toxicity and used only after FDA approval, which is currently under review. The liposome solution and DNA will be aliquoted in individual sterile vials mixed under sterile conditions as described above.

Escalating doses will be studied in this phase I study. Four groups (3 patients each) will be studied sequentially with at least 1 month of observation prior to evaluation of the next group. Patients in each group will receive intratumor injections. Group I will receive 1 injection of ≤0.2 ml. Group II will receive 3 injections at one session of ≤0.2 ml each within the same nodule. Group III will receive 5 such injections at one session, Group IV will receive 5 injections at one session with an increased volume (≤0.4 ml/injection).

Doses will be repeated within each subject for whom the toxicity treatment is with ≤grade II. Dose escalation will begin if three patients show toxicities ≤grade III from the treatment. If one patient displays toxicity >grade II, the treatment will be repeated on three additional patients. If two patients develop toxicity >grade II, the dosage will be reduced. The maximal tolerated dose will be defined as the dose at which two or more patients out of six develop grade III or IV toxicity. The treatment dose will be established at one level below the maximum tolerated dose. Once the treatment dose is defined, an additional four to six patients will be entered at that dose to ascertain the safety of this dose for wider application.

Prior to the injection with the needle in place, gentle aspiration will be applied to the syringe to ensure that no material is injected intravenously. Immediately after the injection procedure, a blood sample will be obtained to check serum enzymes, chemistries, and blood counts, and to analyze for the presence of plasmid DNA in the peripheral blood by PCR. The patients will be observed in the Clinical Research Center for an additional 48 hours, and another blood collection performed as described in Section 9. If there are no complications, the patient will be discharged after 48 hours. Should any abnormalities appear, the patient would be kept for further observation.

6.2 Confirmation of Gene Transfer and Expression

Needle biopsy of the injected nodule will be performed after administration of local anesthesia prior to injection and subsequently just prior to each repeat injection. A portion of this tissue will be processed to obtain DNA for PCR analysis. The remaining tissue will be processed for pathologic analysis and immunohistochemical and/or immunofluorescent staining. If sufficient material can be obtained, RNA PCR analysis will also be performed.

6.3 Analysis of Immune Response

Evidence of gene transfer can also be obtained indirectly by examination of the specific immune response to HLA-B7. The analysis will be performed as follows: two weeks prior to the initial treatment, a blood sample will be obtained to derive lymphocytes which will be immortalized using the Epstein-Barr virus. An aliquot of these cells will be further infected with an amphotropic HLA-B7 retroviral vector, and expression will be confirmed on the cell surface. These cells will subsequently be used in the laboratory as target cells for the cytolytic T cell assay. At no time will these cells be brought into the same building where the patient is being treated. These cells and laboratory experiments will be performed in MSRB II, Room 3560, whereas the patient's clinical treatment will be taking place in the Clinical Research Center of The University of Michigan Medical Center Hospital.

6.4 Repeated Treatment

If no adverse side effects of the treatment are observed, repeated injections will be considered at two-week intervals. Doses identical to the initial treatment regimen will be repeated with similar protocols and observation as described in Section 6.1.

6.5 Postmortem Analysis

The life expectancy of patients who enter this protocol will be limited, in general, less than six months. Important information can be obtained by analysis of tissue postmortem, including presence of DNA in germ line tissue, distribution of DNA and immune function, and potential toxicity. To maximize the information derived from these studies, every effort will be made to perform postmortem analyses. Consent for an autopsy will not be required for entry, but patients and their families will be informed of this aspect of the study and its potential contribution to medical knowledge.

7.0 Schema/Duration

[See table below]

8.0 Dose of Therapeutic Modifications

Patients can receive medications to control any side effects of treatment. Such agents would include acetaminophen (650 mg. q. 4 hrs.), indomethacin (50–75 mg. q. 6 hrs.), or antihistamines as required. Glucocorticoids will not be used in these patients; or if such medication is required, the patient will be removed from the protocol.

Group	*No. of Injections Per Treatment*	*Volume of Injection (ml)*	*Times of Repeated Treatment*	*Total Number of Treatments*
I	1	≤0.2	2 wk.	3
II	3	≤0.2	2 wk.	3
III	5	≤0.2	2 wk.	3
IV	5	≤0.2	2 wk.	4

Parameters	Pre-Study	During Treatment: Day 1	3	5	7	14	21	28	Week 6	10	16
Physical exam	X	X			X	X		X	X	X	
History	X										
Performance Status	X									X	
Tumor Staging and/or Nodule Imaging	X					X		X	X	X	X
Chemistry Survey[1]	X	X	X	X	X	X	X	X	X	X	
Vital Signs	X	X	X	X	X	X	X	X	X	X	X
Weight	X	X	X	X	X	X	X	X	X	X	X
CBC, Diff, Platelet	X	X	X	X	X	X	X	X	X	X	X
PT, PTT	X	X		X		X		X		X	
EKG	X							X		X	
CPK + isoenzymes	X	X	X								
CXR	X							X	X	X	X
U/A and Culture	X										
Hb_sAg, HIV	X										
Brain CT or MRI	X										
Assess for adverse events status	X	X	X	X	X	X	X	X	X	X X	X
PCR on PBL and serum to detect HLA-B7 gene	X	X	X	X		X		X	X	X	X
Tumor biopsy	X				X	X		X	X	X	
Assay for HLA-B7 cytotoxic T cells	X					X		X			X
Assay for HLA-B7 antibody	X					X		X			X
Pregnancy test (if relevant)	X										
Anti-DNA antibodies	X										
ds DNA											
ss DNA											
CT scan of brain (if relevant)	X							X		X	
CT of chest & abdomen	X							X		X	
Quantitate size of lesion	X				X	X		X		X	
ANA, ESR, CH50, CRP	X							X		X	

[1] Routine laboratory tests include uric acid, calcium, phosphate, SGOT, SGPT, alkaline phosphatase, LDH, bilirubin, BUN, creatinine, total protein, glucose, amylase and lipase.

9.0 Study Parameters

9.1 Pre-Treatment (see Table).

The following studies will be performed as summarized in Table 1.

a. Complete physical examination noting in detail the exact size and location of any lesions that exist. The tumor lesion to be treated will be imaged and its dimensions quantitated prior to treatment.
b. Complete chemistry survey including electrolytes, liver function tests, calcium, magnesium, creatinine, BUN, CPK, pancreatic enzymes.
c. CBC, differential count, PT, PTT, platelet count.
d. Urine analysis and culture.
e. Hepatitis screen.
f. HIV titer.
g. Pregnancy test for women.
h. Chest x-ray.
i. Electrocardiogram.
j. Baseline x-rays and nuclear medicine scans to evaluate the status of disease.
k. CT scan or MRI scan of brain.
l. 45 ml of clotted blood for serum storage and 45 ml of anti-coagulated blood for mononuclear cell cryopreservation.
m. Biopsy of tumor, if possible with minimal morbidity.

9.2 Treatment

Patients will be analyzed with routine blood and chemistry analysis. A chest x-ray will be performed each month for six months and every six months thereafter or more often if needed. During the gene transfer protocol, patients will be monitored closely in the Clinical Research Center. Vital signs will be measured every 15 minutes prior to, during, and after the injection for at least two hours or until the patient is stable. A pulse oximeter will be used for on-line measurement of oxygen saturation during injection as well. If the systolic blood pressure drops below 80 mm/Hg or the oxygen saturation drops below 90%, the injection will be terminated immediately.

9.3 Post-treatment

Cutaneous lesions will be evaluated by physical examination, biopsy if feasible, and appropriate imaging scans prior to injection, monthly for 6 months and six-month intervals or as needed to evaluate response to treatment. Serum will be analyzed by PCR for the presence of plasmid, and antibody to HLA-B7 will be evaluated. PBL's will be isolated and analyzed for their ability to lyse HLA-B7 modified autologous EBV-transformed lines. If ANA becomes positive or other signs of autoimmunity appear, evidence of more specific autoantibodies, e.g., anti-Rho, Smith, etc., will be tested and rheumatology consultation will be sought.

9.4 Potential Side Effects and Reporting of Adverse Reactions

Adverse Drug Reactions will be reported to the Drug Information Service, University of Michigan Medical Center, phone 313-936-8200 or 313-936-8251 (available 24 hours), and include all life-threatening events (Grade 4) which may be due to drug administration, all fatal events, or the first occurrence of any *previously unknown* clinical event (regardless of Grade). A written report is to follow within 10 working days to:

Investigational Drug Branch
P.O. Box 30012
Bethesda, Maryland 20824
Phone: 301-496-7957

Data will be submitted to the IRB monthly or upon request. All adverse reactions will be reported to the IRB, even if there is only a suspicion of a drug effect. All side effects will be graded using the standard toxicity sheet described in Section 14, Common Toxicity Criteria.

9.4.1 Potential Risks of Gene Transfer In Vivo

Insertional mutagenesis. The possibility of causing malignancy in cells secondary to the random insertion of the DNA in the genome exists, though this risk is considered low. There is a remote chance that the vector could replicate in the host. Neither finding has been seen in animal studies (~200 mice, rabbits, and pigs); nonetheless, the proposed protocol provides for extensive testing of tumor tissue and blood after injection into the patient. PCR will be performed to monitor for this unlikely event.

The use of aminoglycoside antibiotics. The neomycin resistance gene, which encodes neomycin phosphotransferase (NPT), phosphorylates the 3′ hydroxyl group of the aminohexose I of neomycin and its analogues, inactivating the antibiotic. While amikacin may be inactivated by this enzyme, gentamicin and tobramycin do not contain a hydroxyl at the 3′ position and are not inactivated. Therefore, introduction of the NeoR gene would not exclude the use of aminoglycosides or any other conventional antibiotic that may be needed in the clinical management of these patients.

Although risks to the patient exist in this study, they would appear to be minimal, and the escalating dose nature of this study should minimize the risks to any individual patient. Because these patients have limited life expectancy from their advanced cancer, these risks are thought to be justified, considering the potential therapeutic benefit.

10.0 Off-Study Criteria

Patients will be removed from the study should any grade 3 or 4 toxicity develop which is not easily corrected. The toxicity sheet is included in Section 14.0, Common Toxicity Criteria). If two treatment-related deaths should occur, this protocol will be terminated.

11.0 Evaluation of Results

11.1 Criteria for Response

In this phase I study, the protocol will be considered successful if recombinant gene expression is achieved and an appropriate dosage established for effective gene transfer without toxicity. Confirmation of recombinant gene expression is described below. Additional evidence of successful gene transfer may also be obtained from immunologic analysis. A new CTL or antibody response to HLA-B7 will indicate successful gene expression and is described in Section 11.3. The ability to generate anti-tumor CTL will also be evaluated. Evidence for tumor regression will also be obtained. *Complete tumor response* is defined as the disappearance of all clinical evidence of disease for at least four weeks. *Partial tumor response* is defined as 50% or greater decrease of the sum of the products of perpendicular diameters of lesions lasting at least four weeks with no increase in existing lesions or appearance of new lesions. The response will also be considered positive for gene transfer if cytolytic T cell activity for HLA-B7 is obtained after this injection. Any patient having less than a partial response is considered to be non-responsive to treatment. Tumor dimensions will be assessed by imaging as detailed in Section 9.

11.2 Confirmation of Recombinant Gene Expression

Several independent techniques will be used to evaluate the presence and expression of the recombinant gene *in vivo*. We have used several monoclonal antibodies to HLA-B7 to detect the recombinant gene product *in vivo* by immunohistochemistry. Fluorescence staining of freshly dispersed cells will also be evaluated. The presence of plasmid DNA will be confirmed by PCR of DNA from tumor tissue, peripheral blood lymphocytes, or in autopsy specimen tissue. If sufficient tissue is available, RNA will be isolated and examined for the presence of HLA-B7 mRNA by PCR or S1 nuclease analysis.

11.3 Analysis of Immune Response

Cytolytic T cell activity will be evaluated by incubation of peripheral blood lymphocytes with ^{51}Cr labeled HLA-B7 transduced EBV lines or malignant melanoma cells from the patient. The presence of antibody will be evaluated by FACS analysis of a matched pair of HLA-B7^{+} or HLA-B7^{-} cell lines. In some instances, lymphocytes will be isolated directly from the tumor, expanded in tissue culture, and analyzed for cytolytic function. Tumor biopsies at 7–14 days after treatment will be analyzed by immunohistochemistry. If possible, we will attempt to expand draining lymph node T cells or TIL cells to test their cytologic function. When possible, we will derive autologous cell lines to

be used as targets in a ^{51}Cr release assay. Finally, every attempt will be made to excise tumor tissue prior to treatment for diagnosis, immunohistochemistry, and cryopreservation and to evaluate delayed type hypersensitivity reactions to the tumor before and after treatment.

11.4 Toxicity

Toxic side effects of treatment will be analyzed and classified by the common toxicity criteria (Section 13).

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15.0 Informed Consent (see attached)

University of Michigan Medical School
Department of Internal Medicine
and the Clinical Research Center

CONSENT FORM

(To be read by the Patient and explained to the Patient by his or her Physician).

PROTOCOL: *Immunotherapy of Malignancy by in Vivo Gene Transfer into Tumors*

PRINCIPAL INVESTIGATOR: Gary J. Nabel, M.D., Ph.D.
ASSOCIATE INVESTIGATORS: Alfred Chang, M.D.
Elizabeth G. Nabel, M.D.
Gregory Plautz, M.D.

PROTOCOL NUMBER: __________
DATE: __________
PATIENT NAME: __________
HOSPITAL NO.: __________

INTRODUCTION

We invite you to participate in a research study at the University of Michigan Medical Center. Several general principles apply to all who take part in any experimental studies:

(1) Your participation in this study is voluntary
(2) You may not personally benefit from this study, but knowledge may be gained from it that will benefit others
(3) You may withdraw from the study at any time for any reason without jeopardizing your future care.

The nature of the study, the risks, inconveniences, discomforts, and other information are discussed in the following sections. Please do not hesitate to discuss any questions you have about this study with the physicians who explain it to you.

DESCRIPTION OF TREATMENT OR PROCEDURE TO BE UNDERTAKEN

As you are aware, you have cancer which cannot be cured by medicine, surgery, or radiation. In this study, treatments will be offered that may help to fight this disease in future patients. Because the treatment is experimental, you may not derive any direct benefit from it. The purpose of this study is to determine a safe and effective dose of a new treatment which will attempt to induce tumor regression. Because this is a new, experimental treatment, we will also be observing you to determine the side effects of the therapy. We will also monitor the effects of this treatment on the growth of your tumor.

By using techniques in the laboratory, it is now possible to prepare large amounts of human DNA or genetic material in bacteria. This DNA will be mixed with fat bodies called liposomes, and we plan to transport the mixture into your tumor by needle injection. Once introduced into the tumor, the DNA produces a protein which stimulates tissue rejection. This protein—known as HLA-B7—causes the cells which will contain it to be recognized as "foreign enemy" by your immune system. The purpose of our study is to determine whether this treatment will induce the cells of your immune system, known as lymphocytes, to attack and kill your tumor. This type of therapy which stimulates your lymphocytes is called immunotherapy.

Alternative Therapies

There are no known cures for patients with your disease. Other alternative treatments available to you include radiation or medication to control pain and medical, surgical, or radiation treatment of any reversible complications. Other experimental treatments are under investigation which attempt to stimulate your immune system to reject your tumor, and you can be referred to physicians who are conducting such trials. In contrast to this treatment, other protocols usually require removal of tumor cells or blood cells, laboratory manipulation, and reinjection. In some cases, proteins are injected which can stimulate the immune system. You also have the option to receive no treatment at this time.

Procedures

Before receiving this treatment, you will receive many tests to see if you qualify for this study. These tests will be made either as an inpatient or an outpatient. These tests include: 1) blood tests 2) x-rays of the brain, chest, and abdomen and 3) a blood test for the antibodies to Human Immunodeficiency Virus (HIV), which causes AIDS. If you have antibodies to HIV, you may not participate in this study.

If you qualify for this study, we will inject a solution containing the DNA/liposome complex directly into a tumor nodule. The injections will be made under sterile conditions after providing a local anesthetic (xylocaine), and multiple areas within a single nodule will be injected up to 10 times. Blood samples (between 1–10 tubes) will be obtained daily at first, then weekly for the 1st month, and monthly for the next 6 months. Your blood lymphocytes will be tested for their ability to respond to the HLA-B7 antigen. We will also examine your blood for evidence of toxicity from this treatment.

At different times in the protocol, tumor biopsies will be performed. This procedure involves the injection of a local anesthetic (xylocaine) under sterile conditions, followed by insertion of a needle into the tumor nodule and withdrawal of a sample of the tumor. This procedure will be performed prior to treatment and at intervals of 2–6 weeks up to 4 times.

RISKS AND SIDE EFFECTS

There are potential side effects and risks to this procedure. First, you may experience mild discomfort from needle injections or tumor biopsies. You may have mild discomfort and bleeding from the tumor biopsy. You will be given a local anesthetic to minimize the discomfort. Second, even though the DNA inserted into your tumor is considered harmless to you, events could occur within normal cells that allow them to become cancerous. Labortory studies suggest that this possibility is unlikely. However, this is a new procedure and we do not know whether cells could become abnormal after long periods of time. In animal studies, we have not observed the development of cancer cells in any animals tested. Third, the inserted gene will make a protein that inactivates certain antibiotics. These antibiotics are not usually used to treat infections in humans, and many other antibiotics that are not inactivated will be available and effective in treating any potential bacterial infections.

We emphasize that this procedure, called direct gene transfer, has never been used before in human patients. Because this procedure is new, it is possible that despite our extensive efforts, other unforeseen problems may arise, including the very remote possibility that death may occur.

You will undergo biopsy of tumor and other tissue, if available, on several occasions before and after injection. Blood and tissue specimens will be taken where possible to follow the duration and effects of HLA-B7 expression. If we are successful in this protocol, you will be immunized to the HLA-B7 protein. In the event that you should require an organ transplant, you would not be able to receive an organ from an individual who makes this protein, on average, ~15% of donors.

Follow-Up

After you receive the treatment, you will be discharged from the hospital if you have no other significant medical problems. You will be required to return to the University of Michigan Medical Center for follow-up studies described above. Tests used to decide if your tumor has responded to the therapy will be similar to those you had before beginning the therapy. If your disease recurs after treatment in this protocol, you will be eligible for other protocols and will receive treatment as indicated by your disease or referred elsewhere for such treatment. Because this form of therapy is new, unanticipated side effects that may cause your condition to deteriorate could be encountered. You will be closely monitored for such side effects.

Treatment will continue as long as there is sufficient possibility of response to warrant the risks and side effects encountered. Your physicians feel that the risks of your disease are much greater than the risks of the treatment as outlined above. Furthermore, your physicians have considered your individual situation and have concluded that, at this time, no other therapeutic approaches such as surgery, radiation therapy, or other chemotherapeutic treatments are clinically indicated as being more effective. At some later time, should these alternatives be clinically indicated, they will be discussed with you because this study does not preclude their use.

Other Pertinent Information

1. Confidentiality. When results of a study such as this are reported in medical journals or at meetings, the identification of those taking part is withheld. Medical records are maintained according to current legal requirements, and are made available for review, as required by the Food and Drug Administration or other authorized users, only under the guidelines established by the Federal Privacy Act. A qualified representative of the National Institutes of Health may inspect patient and study records. This procedure may attract attention from the media. We will make every effort to protect your confidentiality. Because of media interest, however, there is a significant chance that information concerning you and your treatment will appear publicly without your consent.
2. Policy regarding research-related injuries. In the unlikely event of physical injury resulting from research procedures, the University will provide first-aid medical treatment. Treatment of injuries or side effects directly related to this experimental treatment will be provided at no cost to you. Additional medical treatment will be provided in accordance with the determination by the University of its responsibility to provide such treatment. However, the University does not provide compensation to a person who is injured while participating as a subject in research.
3. You will not be paid to take part in this study.
4. Outpatient and inpatient studies will be ordered to determine your eligibility for study and whether you have had a response to the treatment. Clinic visits, laboratory tests, x-rays, and scans will not be free. Items which are not covered by insurance which relate to this research protocol will be covered by research grants. The cost of tests and treatments unrelated to this study will be handled as usual and will depend on whether or not you have insurance and what costs your insurance covers. Unfortunately, insurance coverage cannot be guaranteed for all tests and treatments; however, you may discuss this issue with the hospital financial office or your insurance company before you agree to participate.
5. This consent form does not include consent relating to the risks of any surgical procedures. Any surgical procedures performed will require a separate consent form.

Please understand that you are free to withdraw your consent to participate in this study at any time during treatment or follow-up and seek care from any physician with no loss of benefits or disruption in your care.

The team of physicians and nurses providing care in this study are very experienced. You must realize, however, that unforeseeable or unexplained risks are always possible when investigational therapies are undertaken.

If you have questions pertinent to this research, you should contact Gary J. Nabel, M.D., Ph.D., at 313/747-4798.

If you feel that you have a research-related injury, contact Gary J. Nabel, M.D., Ph.D., at 313/747-4798.

The following numbers are for your use if medical problems develop during treatment:

Office: 313/747-4798
Clinical Research Center: 313/936-8090
Voice Mailbox: 313/764-9121 (after office hours)
Doctor or Nurse: 313/936-6266; Paul Watkins, M.D.
(This is a hospital beeper; ask the paging operator to page #9128. Use the following numbers in case of an Emergency Only). (Outside of regular office hours if the paging service won't do.)
Home: 313/———; Dr. Gary J. Nabel

Questions on my rights as a patient may be directed to Ann Munro in the Patient/Staff Relations Office at 313/763–5456.

I have fully explained to the patient, ________________, the nature of the treatment program described above and such risks as are involved in its performance.

____
Physician's Signature

I have been fully informed as to the procedures to be followed including those which are investigational, and have been given a description of the attendant discomforts, risk, and benefits to be expected, and the appropriate alternative procedures. I realize that, since my participation is voluntary, I can refuse

this treatment without in any way prejudicing my future medical care. In signing this consent form, I agree to this method of treatment, and I understand that I will receive the best supportive care even if not receiving this protocol treatment. I also understand that my doctors can stop my treatment on this protocol if they feel the risks in my case have increased, over time, to exceed the potential benefits to me. I understand, also, that if I have any questions at any time, they will be answered. I have received a copy of this consent form.

I am not and will not become pregnant during this study.

I understand that the University will provide first-aid medical treatment in the unlikely event of physical injury resulting from research procedures. Treatment of injuries or side effects directly related to the experimental treatment will be provided at not cost to me. Additional medical treatment will be provided in accordance with the University's determination of its responsibility to do so. The University does not, however, provide compensation to a person who is injured while participating as a subject in research.

I have not engaged in any other research projects with the past six (6) months [ ].

Within the past six (6) months, I have been involved in a study by Dr. ______________________.

I have [ ] have not [ ] been under the care of a physician within the past twelve (12) months.

____
Signature of Patient

____
Date

____
Witness

4/92

16.0 Scientific Abstract

The goal of immunotherapy is to stimulate the immune system by modification of tumor cells or expansion of lymphocytes which respond specifically to tumor antigens. In this study, we will apply techniques of direct gene transfer to enhance immune response against tumors *in vivo*. Patients with advanced cancer who have failed all effective therapy will be treated by injection of a DNA/liposome complex directly within the tumor. DNA encoding an HLA-B7 histocompatibility antigen and the neomycin-resistant gene in a eukaryotic expression vector will be used, and a safe and effective dose to introduce this recombinant gene in HLA-B7$^-$ patients will be established. HLA-B7 expression will be confirmed *in vivo*, and the immune response stimulated by the expression of this antigen will be characterized. We will also determine whether this treatment facilitates tumor regression alone or in combination with other treatment modalities. This gene transfer approach will be analyzed for its efficacy as an anti-cancer treatment. Finally, these studies will allow the development of other approaches, using different recombinant genes or in combination with cytokines or adoptive T cell therapy, to augment tumor immunity. This method to treat malignancy may provide the basis to establish the safety on this general approach, which could be extended to treat a variety of other human diseases.

17.0 Non-technical Abstract

Many types of cancer cannot be cured by traditional medical treatments, including drugs, surgery, or radiation. In this study, an experimental treatment will be offered that may help to fight this disease. We will attempt to induce tumor regression by the introduction of genetic material that directs the synthesis of a protein which stimulates the immune system. The genetic material, DNA, will be introduced directly into the tumor by mixing it with fat bodies, or liposomes, and this mixture will be injected into the tumor. The DNA will be taken into cells and cause them to produce a protein that stimulates tissue rejection. This protein, called HLA-B7, causes cells which contain it to be recognized as foreign by the immune system. The goal of the treatment is to stimulate the immune system to attack and kill the tumor. In this study, we will determine a safe and effective dose to administer the DNA/liposome complex. Increasing amounts of this complex will be used in different patient populations. If no side effects are observed, repeated treatments will be instituted. The expression and nature of the immune response will also be characterized. This treatment may provide a therapeutic effect in cancer and could be applied to the treatment of other diseases.

NOTE ADDED IN PROOF: The Response to the Points to Consider will be published in the next issue (volume 3, number 5).